Extracellular vesicle miR-7977 is involved in hematopoietic dysfunction of mesenchymal stromal cells via poly(rC) binding protein 1 reduction in myeloid neoplasms

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Supplementary Materials

Supplementary Table S1. Phenotype of MSCs derived from Lymphoma stage 1, AML and MDS patients

MSC ID	Age (years)	Diagnosis	Passage number	SD	Percentage of CD105+ cells	Percentage of CD166+ cells	Karyotype of MSCs
1	80	AML (M4)	5	2.6 ± 1.5	94.4%	88.0%	46, XY
2	44	AML (M2)	5	4.0 ± 0.8	99.0%	96.0%	46, XX
3	77	AML (M5)	5	4.0 ± 0.5	97.0%	84.0%	46, XY
4	70	AML/MLD	7	7.2 ± 1.5	85.0%	90.0%	46, XY
5	79	AML/MLD	7	7.0 ± 0.8	91.0%	92.0%	46, XX
6	64	RAEB-2	5	5.2 ± 1.2	89.0%	88.0%	46, XY
7	76	RCUD	8	7.1 ± 1.4	80.0%	85.0%	46, XX
8	72	RCUD	5	4.6 ± 1.1	93.0%	91.0%	46, XX
9	75	RCMD	5	3.6 ± 1.1	96.0%	90.0%	46, XX
10	40	MALT lymphoma	5	4.0 ± 0.8	95.0%	94.0%	46, XX
11	44	Folicular lymphoma	5	4.0 ± 0.8	92.0%	92.0%	46, XX
12	55	MALT lymphoma	5	4.0 ± 0.8	96.0%	91.0%	46, XY

SD, saturation density (number of cells, 10^{-4} per cm^2); AML: acute myeloid leukemia; AML/MLD: AML with multilineage dysplasia; RA: refractory anemia; RAEB: RA with excess blast. MALT lymphoma; Extranodal marginal zone lymphoma of mucosa-associated lymphoid tissue

Supplementary Table S2. The profiles of the patinets, the sample ID and MSC ID in this study.

Sample ID	MSC ID	Sex	Age	WHO	IPSS	IPSS-R	WBC	RBC	Hb	Plt
1	NA	F	73	Hodgkin Lymphoma	NA	NA	4900	435	12.7	21.8
2	NA	M	54	CNS lymphoma	NA	NA	9100	554	16.6	20.7
3	10	F	40	MALT lymphoma	NA	NA	7200	435	12.8	37.3
4	11	F	44	Folicular lymphoma	NA	NA	6000	398	12.2	32
5	NA	F	59	MALT lymphoma	NA	NA	7000	450	13.4	36.2
6	NA	F	73	MALT lymphoma	NA	NA	4900	435	12.7	21.8
7	12	M	55	MALT lymphoma	NA	NA	9100	554	16.6	20.7
8	NA	F	41	Hodgkin Lymphoma	NA	NA	7200	435	12.8	37.3
9	NA	F	59	MALT lymphoma	NA	NA	7000	450	13.4	36.2
10	NA	M	74	RARS	Int-1	Very low	3900	287	10.6	21.9
11	NA	F	55	RCUD	Low	Very low	3100	456	13.3	4.5
12	NA	F	54	RCUD	Low	Very low	3100	460	13.3	4.5
13	NA	F	53	RCUD	Low	Very low	3100	506	13.3	4.5
14	NA	M	73	RCUD	Low	Low	3900	287	10.6	21.9
15	NA	M	72	RCUD	Low	Low	3900	287	10.6	21.9
16	NA	M	61	RCMD	Low	Low	2000	217	7	23.2
17	NA	M	82	RCMD	Int-1	Intermediate	1800	230	7.6	11.2
18	NA	M	61	RCMD	Low	Low	2000	217	7	23.2
19	NA	M	60	RCMD	Low	Low	2000	217	7	34.9
20	NA	M	82	RCMD	Int-1	Intermediate	1800	230	7.6	11.2
21	NA	M	59	RCMD	Int-1	Low	2000	217	7	23.2
22	NA	M	71	RAEB-1	Int-1	High	1300	254	9.4	6.9
23	NA	F	60	RAEB-1	Int-2	Very high	600	217	6.6	4.2
24	NA	M	70	RAEB-1	Int-1	High	1300	254	9.4	6.9
25	NA	F	61	RAEB-2	Int-2	Very high	600	217	6.6	4.2
26	NA	M	72	RAEB-2	Int-2	High	1300	254	9.4	6.9
27	NA	F	59	RAEB-2	High	Very high	600	217	6.6	4.2
28	NA	M	43	MDS/AML	High	High	1300	326	10.1	6.5
29	NA	F	61	MDS/AML	High	Very high	400	223	6.6	2.7
30	NA	M	76	MDS/AML	High	Very high	2100	256	9.4	2.4
31	NA	F	60	MDS/AML	High	Very high	500	260	6.8	3.7
32	NA	M	75	MDS/AML	High	Very high	2100	256	9.4	2.4
33	NA	F	60	MDS/AML	High	Very high	500	260	6.8	3.7
34	NA	M	58	AML M2	NA	NA	72400	279	7.8	4.2
35	NA	F	63	AML M1	NA	NA	69200	333	8.6	5.5
36	NA	M	57	AML M2	NA	NA	42400	279	7.8	4.5
37	NA	M	29	AML M2	NA	NA	4300	348	7	6
38	NA	F	64	AML M0	NA	NA	59200	303	8.9	6.5
39	NA	F	36	AML M2	NA	NA	40700	242	8	6.8
40	NA	M	30	AML M4	NA	NA	3300	308	9.1	6.3
41	1	M	80	AML M4	NA	NA	ND	ND	ND	ND
42	2	F	44	AML M2	NA	NA	ND	ND	ND	ND
43	3	M	77	AML M5	NA	NA	ND	ND	ND	ND
44	4	M	70	AML/MLD	NA	NA	ND	ND	ND	ND
45	5	F	79	AML/MLD	NA	NA	ND	ND	ND	ND
46	6	M	64	RAEB-2	High	High	ND	ND	ND	ND
47	7	F	76	RCUD	Low	Low	ND	ND	ND	ND
48	8	F	72	RCUD	Int-1	Intermediate	ND	ND	ND	ND
49	9	F	75	RCMD	Int-2	High	ND	ND	ND	ND

AML: acute myeloid leukemia; AML/MLD: AML with multilineage dysplasia; NA: not applicable, ND; not determine

Supplementary Table S3. Primer set IDs for genes examined using Taqman quantitative real-time PCR (qRT-PCR)

Gene name (Gene Alias)	Taqman Assays ID
Human stem cell factor (SCF)	Hs00241497_m1
Human thrombopoietin (TPO)	Hs00171249_m1
Human Flk2/Flt3 ligand (FLT3LG)	Hs00181740_m1
Angiopoietin 1 (ANGPT1)	Hs00181613_m1
Interleukin-6 (IL-6)	Hs0985639_m1
Jagged-1 (JAG1)	Hs00164982_m1
N-cadherin (N-CAD)	Hs00983056_m1
Dicer1 (DICER1)	Hs00229023_m1
Transferrin receptor 1 (TFR1)	Hs00951083_m1
Ferritin, light polypeptide (FTL)	Hs00830226_gH
Ferritin mitochondrial (FTMT)	Hs00893202_s1
Aconitase 1, soluble (IRP1)	Hs00158095_m1
Iron-responsive element binding protein 2 (IRP2)	Hs00386293_m1
Glyceraldehyde 3-phosphate dehydrogenase (GAPDH)	Hs9999905_m1
Human ribosomal protein S18 (18S)	Hs9999901_s1

Supplementary Table S4. Real-time SYBR® Green PCR, primer set IDs

Gene symbol	Primer set IDs
JAG1	PPH06022B
KITLG	PPH00507G
FLT3LG	PPH06324A
SMPD2	PPH09509A
18S rRNA	PPH05666E
GAPDH	PPH00150F

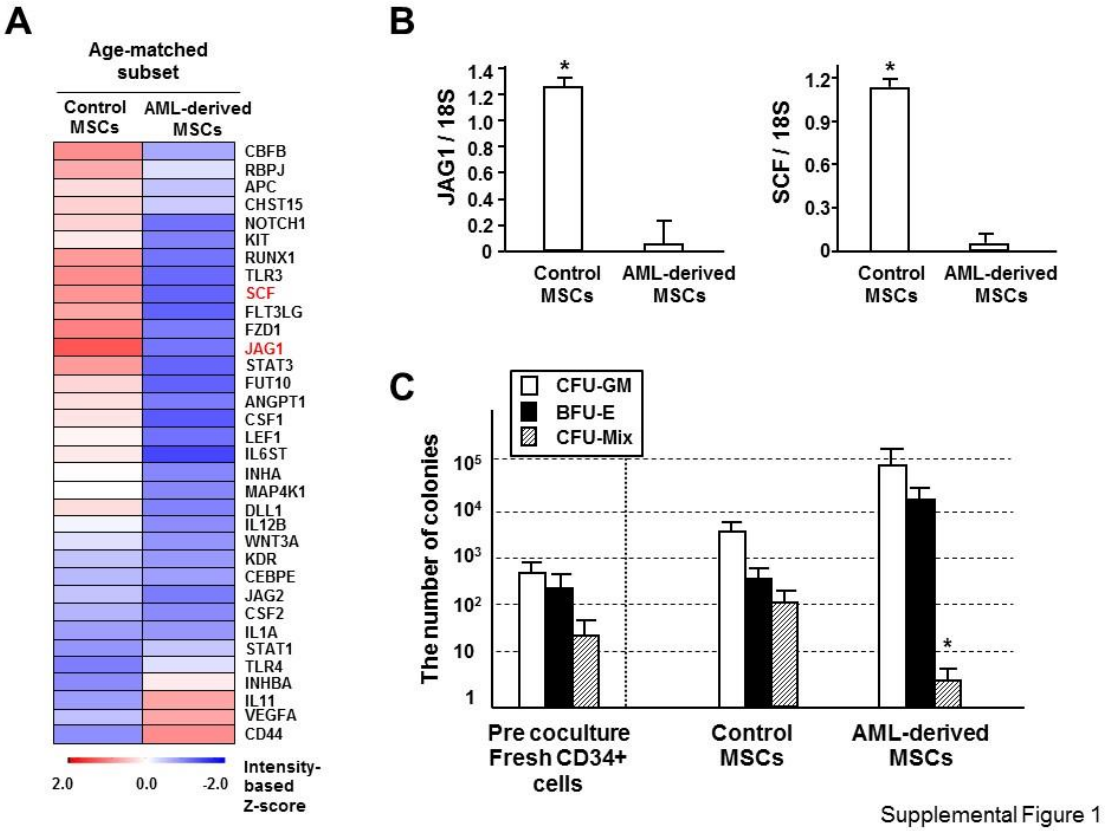
Supplementary Table S5. miScript Primer assay, primer set IDs

miScript Primer	Primer set IDs
human miR-4286	MS00021371
human miR-7977	MS00048930
human miR-8073	MS00048769
Hs_SNORD61	MS00033705
Hs_RNU6-2	MS00033740

Supplementary Table S6. miRNA mimics and IDs

miRNA mimic	IDs
Syn-hsa-miR-7977 miScript miRNA Mimic	MSY0031180
Syn-hsa-miR-4286 miScript miRNA Mimic	MSY0016916
Syn-hsa-miR-8073 miScript miRNA Mimic	MSY0031000
AllStars Negative Control siRNA	1027280
miR-7977 miScript target protector for JAG1 (5'-GGTAGTTTCTGTGGTTGGCTGGGAAATCGAGTGCCGCATC-3')	MTP0076722
miR-7977 miScript target protector for PCBP1 (5'-TCTGTTCAGCTGTTAATGCTGGGATCCATATTTAGTTTTA-3')	MTP0076708
Neg. Control miScript Target Protector	MTP0000002

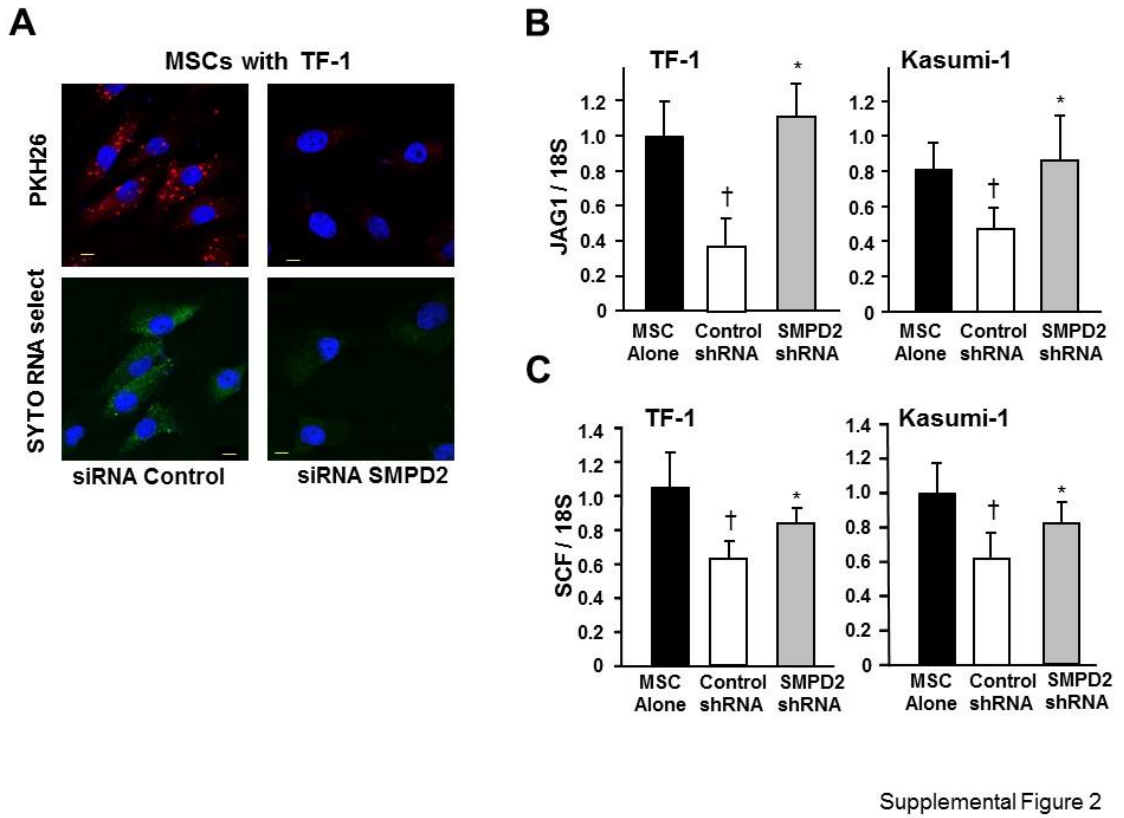
Supplementary Figures and Legends



Supplementary Figure S1.

Comparative analysis of mRNA expression between age-matched HV- and AML-derived MSCs. (A) Comprehensive analysis of hematopoietic factors in age-matched MSCs (44-years-old) by qRT-PCR array. Heatmap shows the expression of hematopoietic factors in MSCs derived from control (ID 11) and AML M2 (ID 2) patients (*Online Supplementary Table S1*). (B) The expression levels of JAG1 and SCF mRNAs were further confirmed by qRT-PCR. *p < 0.01 control vs. AML. Data represent three independent experiments, each done in triplicate. Y-axis indicates the expression ratio of target mRNA relative to 18S rRNA internal control. *p < 0.01 control vs. AML. (C) Clonogenic assay after coculture with human stromal cells. Y-axis indicates the number

of colonies after *ex vivo* coculture of 2 × 10^4 BM CD34+ cells on MSC layer. X-axis indicates the individual MSCs derived from control and AML patients. Pre-coculture indicates the number of colonies derived from fresh BM CD34+ cells. MSCs derived from HV were used as normal control. *$p < 0.01$, colony-forming units (CFU)-Mix: primary AML-derived MSCs vs. normal control-derived MSCs (Student's t-test, two-tailed). BFU-E, burst forming units of erythroid; CFU-GM, CFU-granulocyte/monocyte; CFU-Mix, CFU-mixed. Results are expressed as means ± SD. Similar results were obtained in 3 independent experiments, performed in triplicate.

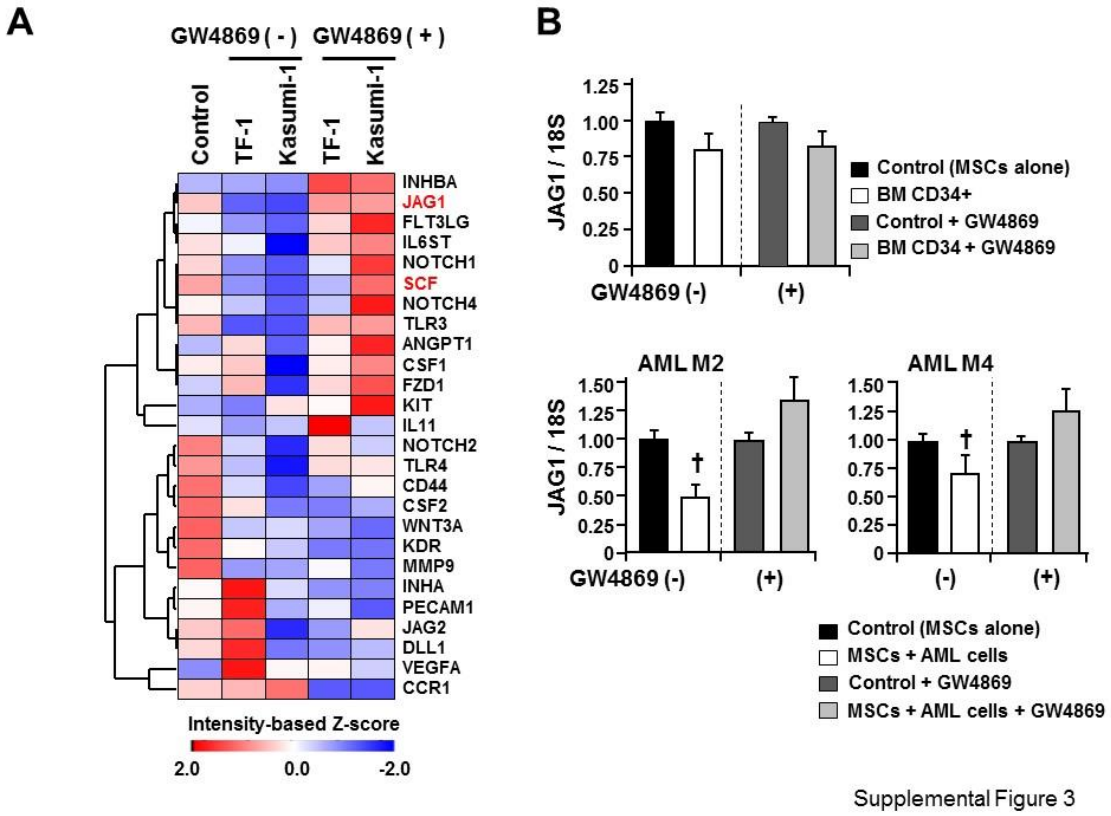


Supplemental Figure 2

Supplementary Figure S2.

EV transfer assay was conducted after transduction with control, SMPD2 siRNA or SMPD2 shRNA into hematopoietic cells. (A) MSCs were cocultured in non-contact system with TF-1 cells, and PKH26 or SYTO RNAselect signal was visualized by confocal microscopy. Cells were fixed with 4% paraformaldehyde for PKH26 or fixed with methanol for SYTO RNAselect. Scale bars: 10 μm. Changes in JAG1 (B) and SCF (C) mRNA levels in MSCs cocultured with TF-1 or Kasumi-1 cells transduced with SMPD2 shRNA or negative control shRNA expression vector (Supplementary Methods) were examined by qRT-PCR. MSCs were cultured in serum-free StemPro®-34 medium to avoid the effects of EVs. Y-axis indicates fold change relative to the control (=1.0) after normalization to 18S expression level. Data shown are from one representative experiment of three showing similar results, each done in sextuplicate. Results are

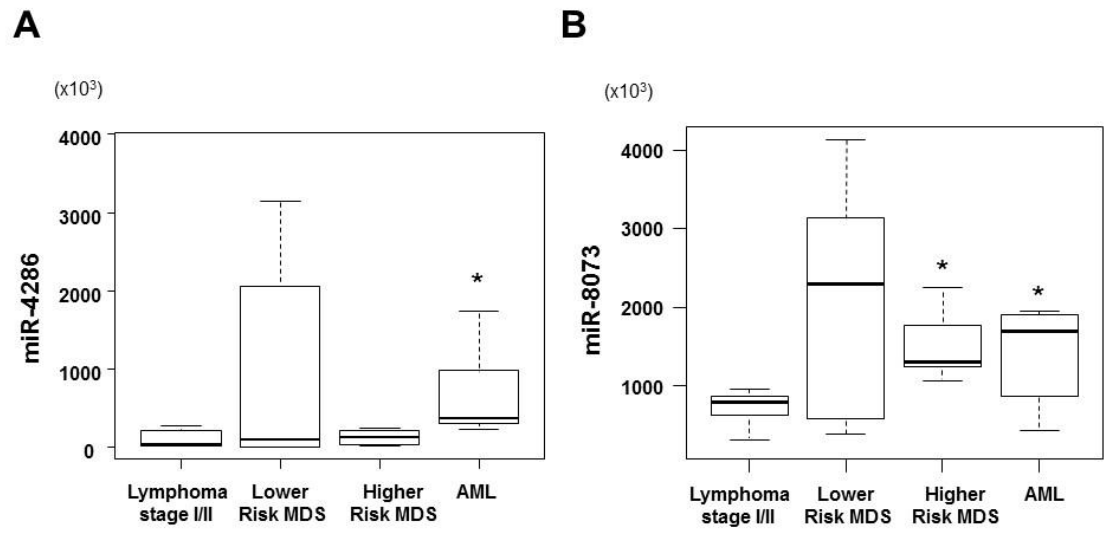
expressed as means ± SD. $^{\dagger}p < 0.05$, HV-derived MSCs without coculture vs. HV-derived MSCs cocultured with AML cells transduced with control shRNA (Student's t-test, two-tailed). $*p < 0.05$, HV-derived MSCs cocultured with AML cells transduced with control shRNA vs. HV-derived MSCs cocultured with AML cells transduced with SMPD2 shRNA (Student's t-test, two-tailed).



Supplementary Figure S3.

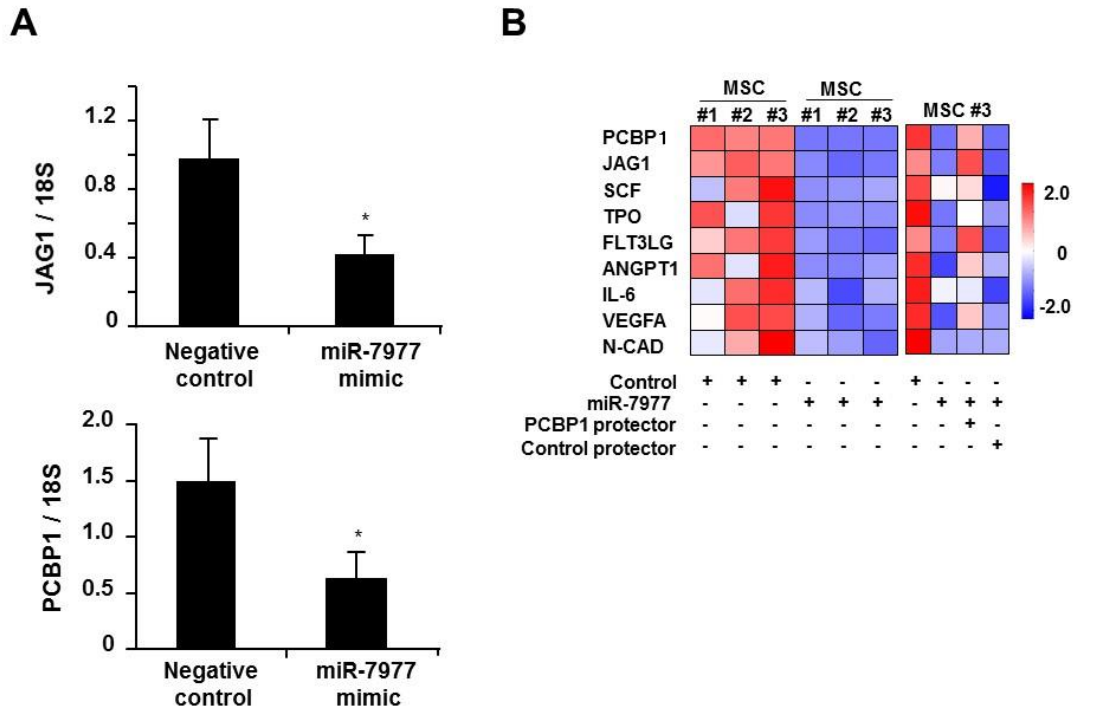
qRT-PCR analysis of JAG1 and SCF mRNA expression in MSCs in the presence or absence of EV inhibition. (A) The effect of leukemic cells with or without GW4869 on the expression of hematopoietic factors in MSCs in the presence of non-contact leukemic cells. Changes in gene expression of hematopoietic factors in MSCs were analyzed by qRT-PCR array. Heatmap indicates the change in expression of target genes as compared with those in control MSCs. Inhibitor (+) indicates the exposure to 3 μM GW4869, and Inhibitor (-) indicates the addition of vehicle control. (B) Change in JAG1 mRNA expression in MSCs cocultured with primary normal BM or AML CD34+ cells in serum-free StemPro®-34 medium. Y-axis indicates the fold change relative to the control (=1.0) after normalization to 18S expression level with or without GW4869. Inhibitor (+) indicates the exposure to 3 μM GW4869, and Inhibitor (-) indicates the

addition of vehicle control. Data shown are from one representative experiment of three showing similar results, each done in quadruplicate. Results are expressed as means ± SD. $^{\dagger}p < 0.05$, HV-derived MSCs without coculture vs. HV-derived MSCs cocultured with AML cells (Student's t-test, two-tailed). $*p < 0.05$, HV-derived MSCs without coculture in the presence of GW4869 vs. HV-derived MSCs cocultured with AML cells in the presence of GW4869 (Student's t-test, two-tailed).



Supplementary Figure S4.

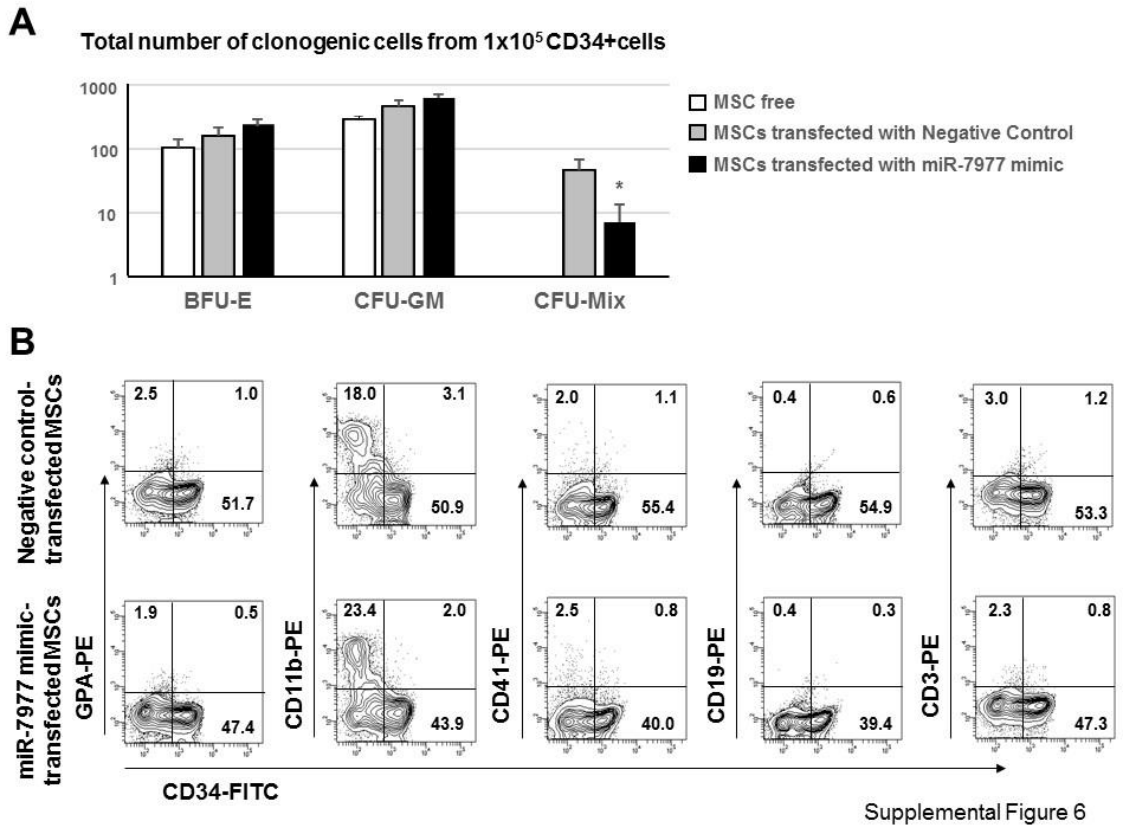
EV miR-4286 and miR-8073 in BM fluid were analyzed by qRT-PCR. The miR-4286 and miR-8073 mimics were used as internal positive controls during miRNA quantification. Y-axis indicates the copy number of miR-4286 (A) or miR-8073 (B) in 1 mL of plasma obtained by BM aspiration. Lymphoma stage I/II (control, n=9), lower risk MDS (n=10), higher risk MDS (n=8) and AML (n=13) patients. *$p < 0.05$, the copy number of miR-4286 or miR-8073 in higher risk MDS or AML vs. the copy number of miR-4286 or miR-8073 in control (ANOVA, followed by Dunnett's multiple comparison tests).



Supplemental Figure 5

Supplementary Figure S5.

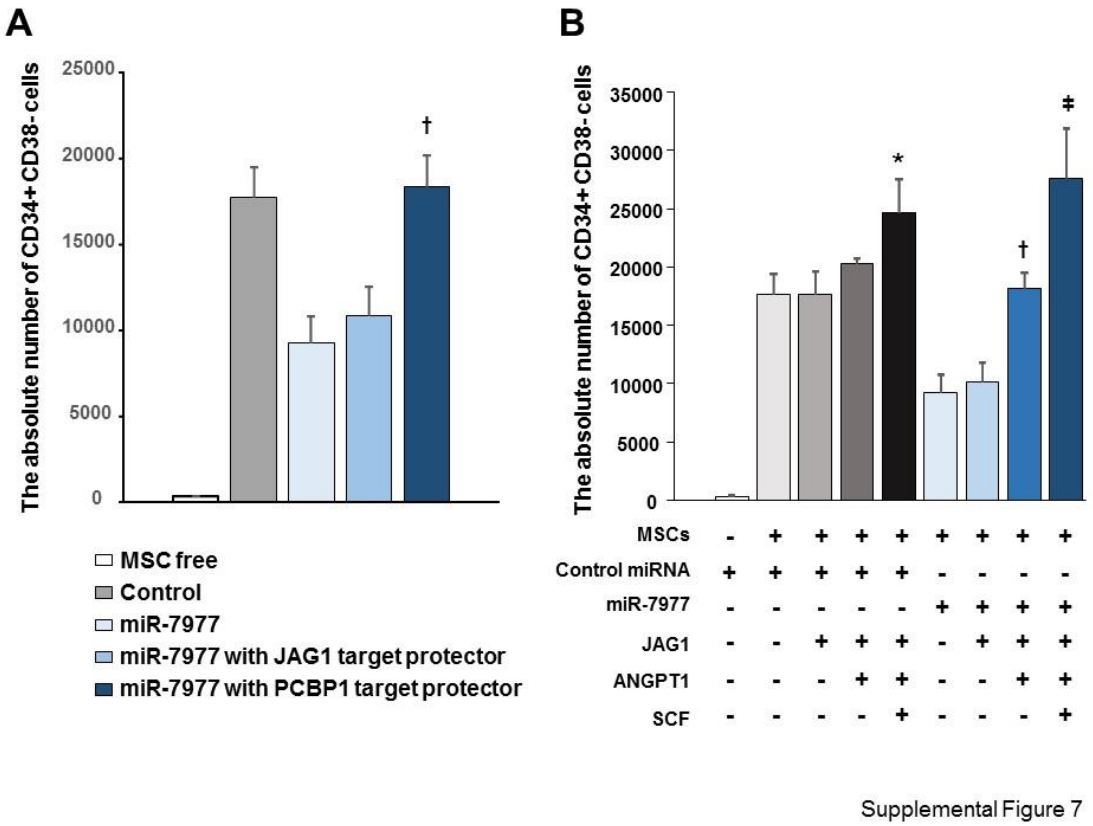
The effect of miR-7977 on mRNA expression. (A) The effect of 5 nM miR-7977 mimic that was transferred into normal MSCs on the mRNA expression of JAG1 (upper panel) and PCBP1 (lower panel) was analyzed by qRT-PCR. *p < 0.05 MSCs transfected with negative control vs. MSCs transfected with miR-7977 mimic (Student's *t*-test). Y-axis indicates the expression ratio of target mRNA relative to 18S rRNA internal control. Data shown are from one representative experiment of two showing similar results, each done in triplicate. (B) The mRNA levels of multiple growth factors after transfection of negative control and miR-7977 mimic with or without miScript Target Protector for PCBP1 were analyzed by qRT-PCR. #1, #2 and #3 indicate lot number of normal MSCs. N-CAD, N-cadherin. Data shown are from one representative experiment of three showing similar results.



Supplementary Figure S6.

Expression of surface antigens on primitive hematopoietic cells that were generated after coculture of BM CD34+ cells for one week. (A) The number of clonogenic cells after coculture of BM CD34+ cells with or without MSCs transfected with negative control miRNA or miR-7977 mimic for one week in the presence of cytokines. *$p < 0.05$, MSCs transfected with negative control miRNA vs. MSCs transfected with miR-7977 mimic (Mann-Whitney U-test). (B) The X-axis indicates CD34 expression labelled with FITC-conjugated monoclonal antibody. The Y-axis indicates Glycophorin A (GPA), CD11b, CD41, CD19 or CD3 expression labeled with PE-conjugated monoclonal antibody. Positivity for a surface antigen was defined using the isotype control monoclonal antibody. Upper panels, MSCs transfected with negative control miRNA;

Lower panels MSCs transfected with miR-7977 mimic. Data are from 2 independent experiments, each done in triplicate.

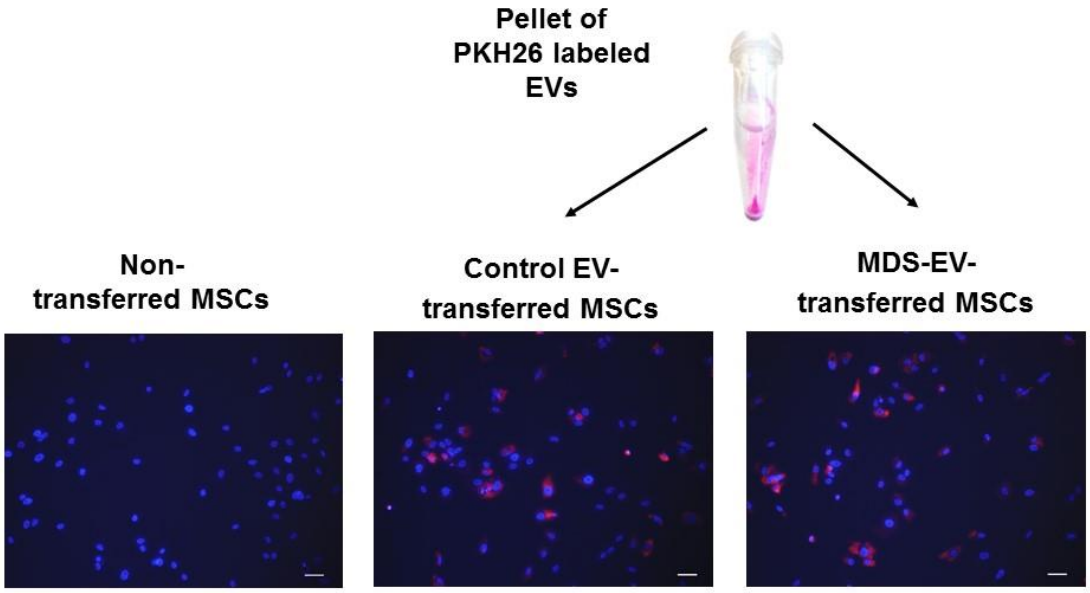


Supplemental Figure 7

Supplementary Figure S7

The effect of target protector and excess growth factors on hematopoietic-supporting capacity of miR-7977 mimic-transfected MSCs. (A) The effect of JAG1 or PCBP1 target protector. BM CD34+ cells were cocultured with target protector or miR-7977 mimic-transfected MSCs for one week. The Y-axis indicates absolute number of CD34+CD38- positive cells. MSC free: CD34+CD38- cells among cultured hematopoietic cells without MSCs. *p < 0.01 miR-7977 mimic vs. miR-7977 mimic with PCBP1 target protector. (B) The effect of excess growth factors including JAG1, ANGPT1 or SCF. 2 μg/ml human JAG1 Fc Chimera, human SCF expression vector or human ANGPT1 expression vector was used for coculture. The Y-axis indicates absolute number of CD34+CD38- positive cells. *p < 0.01 negative control miRNA vs. negative control miRNA with human JAG1, ANGPT1 and SCF. †p < 0.01 miR-7977 vs.

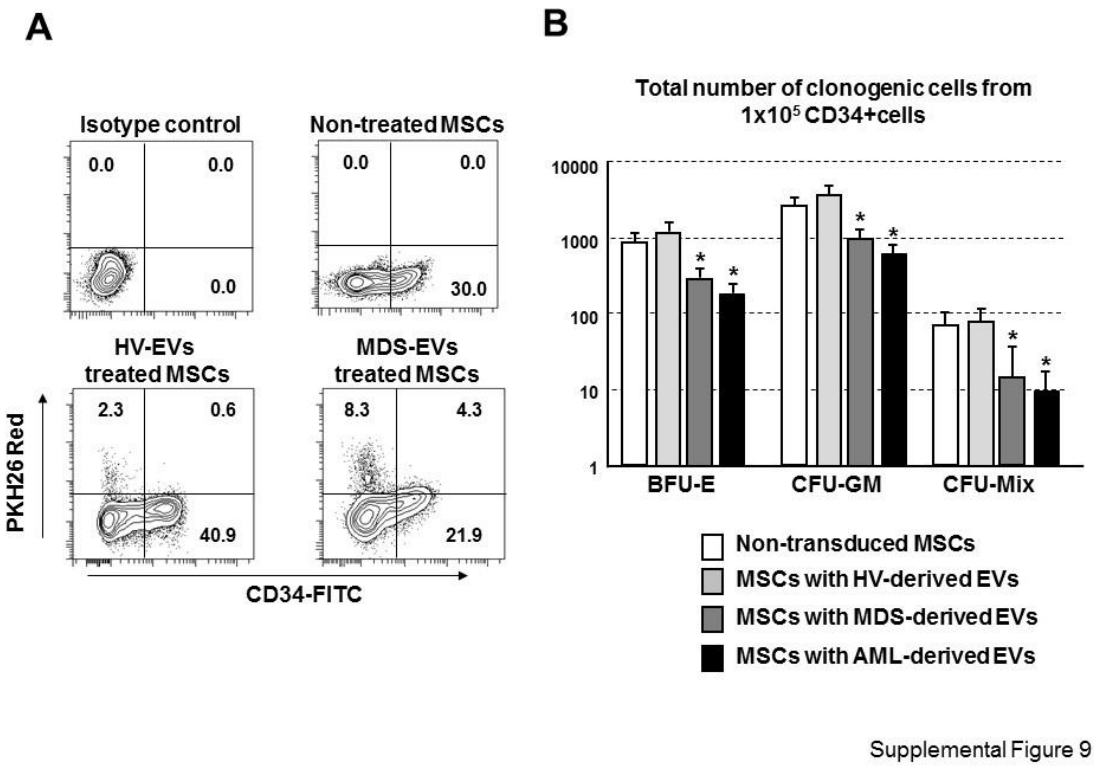
miR-7977 with human JAG1 and ANGPT1. ǂp < 0.01 miR-7977 with human JAG1 and ANGPT1 vs. miR-7977 with human JAG1, ANGPT1 and SCF.



Supplemental Figure 8

Supplementary Figure S8.

Transfer of PKH26-labelled EVs into MSCs. Upper diagram indicates PKH26-labeled EVs 2 hr after addition of ExoQuick-TC. Left panel: Non-transferred control. Middle panel: MSCs after transfer of lymphoma (control)-derived EVs. Right panel: MSCs after transfer of MDS-derived EVs. The nuclear staining was conducted using Hoechst 33342 (Invitrogen, Tokyo, Japan). Cells were visualized using a Biozero BZ-8000 laser scanning microscope (KEYENCE, Laboratories, Tokyo, Japan). Scale bars: 10 μm.



Supplemental Figure 9

Supplementary Figure S9.

Analysis of hematopoietic cells after coculture of hematopoietic cells with EV-transferred MSCs for one week. (A) Expression of CD34 in *ex vivo* hematopoietic cells cocultured with MSCs harboring PKH26 red-labelled EVs. Bottom left panel: Expression of CD34 in hematopoietic cells cocultured with MSCs harboring HV-derived EVs. Bottom right panel: Expression of CD34 in hematopoietic cells cocultured with MSCs harboring MDS-derived EVs. AML/MDS-derived EVs contained abundant miR-7977. The X-axis indicates FITC-labelled CD34, and the Y-axis indicates PKH26 red. PKH26 red was used to determine the efficiency of EV transfer into MSCs before coculture. Data shown are from one representative experiment of three showing similar results. (B) The number of clonogenic hematopoietic cells after coculturing with the indicated MSCs for one week. The X-axis indicates the number of clonogenic cells from 1 x 10^5 CD34+ cells.

X-axis indicates the types of clonogenic cells. *p < 0.05, MSCs with HV-derived EVs vs. MSCs with AML/MDS-derived EVs (Mann-Whitney U-test). Data shown are from one representative experiment of two showing similar results, each done in triplicate.

Supplementary Methods

Analysis of mRNA and miRNA expression

For reverse transcription, total RNA was prepared from cells using the Trizol reagent according to the manufacturer's instructions (Invitrogen, Waltham, MA). Total RNA (1 μg) was reverse transcribed with the SuperScript® VILO™ cDNA Synthesis Kit (Invitrogen), RT^2 First Strand Kit (QIAGEN, Hilden, Germany) and miScript Ⅱ RT Kit (QIAGEN) for Taqman PCR, SYBR® Green PCR and miScript SYBRR Green PCR (QIAGEN), respectively. Taqman Assay IDs and primer set IDs for real-time SYBR® Green PCR and miScript Primer assay are indicated in *Online Supplementary Tables S3, S4 and S5*, respectively. Quantitative real-time PCR (qRT-PCR) was performed in triplicate using the ABI PRISM®7300 Sequence Detection System (Applied Biosystems, Waltham, MA) in a 25 μL reaction volume. RT^2 Profiler PCR Arrays (Hematopoietic Stem Cell and Hematopoiesis PCR array: Version 4.0 PCR Array, #PAHS-054Z; QIAGEN) were conducted according to the manufacturer's instructions using five housekeeping genes for normalization.

Transduction of shRNA against SMPD2 into leukemic cell lines

The negative control vector, pSINsi-hU6**,** was purchased from Takara Bio (Tokyo, Japan). SMPD2 (NCBI accession AJ250460) specific oligo DNA (CAACAAGTGTAACGACGAT) was screened by Takara Bio (Tokyo, Japan) and ligated into the cloning site of pSINsi-hU6 to construct pSINsi-hU6-SMPD2 shRNA. Retroviral supernatant was produced from phoenix-AMPHO cells (American Type Culture Collection, Manassa, VA) after transfection of purified plasmid DNA (Qiagen, Tokyo, Japan) with Lipofectamine 2000 transfection reagent (Life Technologies, Carlsbad, CA). The viral supernatants containing SMPD2 shRNA and negative control (empty pSINsi-hU6 vector) were used to infect leukemic cell lines cultured on

Retronectin® (Takara Bio, Tokyo, Japan). The level of SMPD2 mRNA after transduction of shRNA was analyzed by real time RT-PCR.

Coculture of BM CD34+ cells with human MSCs

Four hundred thousand primary human MSCs were plated in a 25-cm^2 flask and cultured until they reached over 90% confluency. In some experiments, 10 μg of pCA-ANG1 (ANGPT1 expression vector)[35] and pCMV6-entry human SCF (KITLG transcript variant b) (OriGene Inc, Rockville, MD) were transfected into MSCs using Lipofectamine 2000 transfection reagent (Life Technologies) 3 days before coculture. On the first day of coculture, the MSCs were washed five times with phosphate-buffered saline (PBS) before adding the CD34+ fraction of normal BM or AML/MDS cells. These CD34+ cells were seeded on a monolayer of human MSCs in 5 mL of serum-free StemPro®-34 medium (Life Technologies) supplemented with 50 ng/mL human TPO, 10 ng/mL human SCF, 50 ng/mL human FLT3LG and 100 ng/mL human DLL4 with or without 2 μg/mL JAG-1 Fc Chimera (all R&D Systems, Minneapolis, MN), and maintained at 37°C under 5% CO_2. All hematopoietic cells that had cocultured with MSCs were collected as previously described with some modification; we harvested the cocultured cells at 2 weeks. To avoid contamination of the hematopoietic cell suspensions with MSCs, a 30-minute adhesion procedure was performed at 37°C as previously described.[22, 23]

Contact and non-contact culture systems

Contact and non-contact culture systems were conducted using Polyester Membrane Transwell Clear Inserts and Companion Plates (BD Biosciences, San Jose, CA; pore size: 0.4 μm, pore density: $1x10^8/cm^2$, 12 well) as reported previously [24]. For the contact culture, 5 x 10^4 human MSCs were plated on the back of the insert membrane.

For the non-contact culture, MSCs were cultured in companion plates for two days. Primary leukemic cells or myeloid leukemia cell lines were resuspended in StemPro®-34 in the presence of SCF, TPO, FLT3LG, DLL4 and IL-3 (R&D Systems). 1x 10^5 leukemic cells were added to each culture insert. After 14 days of culture at 37°C and 5% CO_2, MSCs were collected.

Clonogenic analysis of cocultured hematopoietic cells

The clonogenic assay was performed using MethoCult GF H4434V (Stem Cell Technologies, Vancouver, Canada). The total number of burst forming units of erythroid (BFU-E), colony-forming units (CFU)-granulocyte/monocyte (GM) and CFU-mixed (Mix) cells was evaluated [23].

Immunophenotyping of MSCs and *ex vivo* cultured hematopoietic cells

Aliquots of cells were stained with FITC- and/or phycoerythrin (PE)-conjugated monoclonal antibodies including the isotype control antibodies (BD Biosciences, San Jose, CA). Cells were incubated with FITC-conjugated anti-CD34 antibody, and PE-conjugated anti-CD38, anti-CD41, anti-Glycophorin A (GPA) (Immunotech), anti-CD19, anti-CD11b, anti-CD3 (Dako Japan, Kyoto, Japan), anti-CD105 (Ancell, Bayport, MN, USA) or anti-CD166 (BD Bioscience, Tokyo, Japan) antibody at 4°C for 60 min, and then washed twice with PBS containing 0.1% BSA. The cells were analyzed by flow cytometric analysis using FACSCanto (Becton Dickinson, Mountain View, CA) and dead cells were gated out by propidium iodide (PI) staining.

***In vivo* repopulating assay**

Cocultured CD34+ cells were injected through the lateral tail vein into NOD/SCID/IL2R γchain^{null} (NSG) mice that had been irradiated with 300 cGy (Softex, Tokyo, Japan) 24

hours prior to cell infusion. Peripheral blood (PB) mononuclear cells (MNCs) were analyzed at 6, 12 and 24 weeks after transplantation.

Transmission electron microscopy and western blotting of EV proteins

EVs were confirmed by transmission electron microscopy using the negative staining method. EVs were fixed with 4% paraformaldehyde soon after preparation loaded on corrosion/carbon-coated copper grids (Nisshin-EM Co., Ltd. Shizuoka, Japan), incubated for 20 minutes and washed with PBS. These samples were further fixed with 2.5% glutaraldehyde and washed in double distilled H_2O. Fixed samples were stained with 2% uranyl acetate, and the grids were let to air dry. Samples were examined at an acceleration voltage of 80 kV with a JEM-1400 transmission electron microscope (JEOL Ltd., Tokyo, Japan). EV proteins were analyzed by immunoblotting analysis using anti-CD63 (System Biosciences, Mountain View, CA), anti-ALIX (ab11766) and anti-TSG101 (ab125011) antibodies (Abcam®, Tokyo, Japan).

EV labeling with PKH26 (IS CHANGE OK?)

EVs were labelled with PKH26, according to the manufacturer's protocol, with some modifications. Briefly, the BM fluid was centrifuged at 3,000 g for 15 min to remove cells and apoptotic bodies. Subsequently, the sample was passed through a 0.45 μm pore size Millipore Hydrophilic Durapore filter (Merck Millipore, Tokyo, Japan). One mL of resulting filtrate was mixed with staining solution containing 1 mL Diluent C and 4 μl PKH26. The mixed solution was incubated for four minutes and the appropriate volume of ExoQuick-TC was added. After incubation at 4°C for 2 hr, the mixed solution was centrifuged at 1,500 g for 30 min and the supernatant was removed. The pellet was used as PKH26-labelled EVs after centrifugation at 1,500 g for 5 min to remove residual solution.

Quantification of EV miRNA

Total RNA in EV was prepared using the Total Exosomal RNA Protein Isolation Kit (Invitrogen). For reverse transcription, 10 μl of EV RNA was reverse transcribed with the miScript Ⅱ RT Kit (QIAGEN). miScript miRNA mimics, SNORD61 or RNU6-2 was used as internal positive control during miRNA quantification.

Transfection of siRNA or miRNA mimics and miScript Target Protectors into MSCs

The miRNA mimics (QIAGEN) were utilized to confirm the target of each miRNA (*Online Supplementary Table S6*). Transfection of siRNA or miRNA mimic (5 nM) was conducted using Lipofectamine™ RNAiMAX Transfection Reagent (Life Technologies) or HiPerFect Transfection Reagent (QIAGEN). In some experiments, cotransfection of miR-7977 miScript Target Protector for JAG1, PCBP1 or negative control (250 nM) with miR-7977 mimic was conducted. The mRNA expression was analyzed by qRT-PCR 3 days after miRNA transfer.

Labeling of miR-7977 with Cy5

miR-7977 was labeled with Cy5 using Label IT® miRNA Labeling Kit (Mirus, Madison, WI) according to the manufacturer's instructions. Primary AML cells were transfected with 50 nM of Cy5-labeled miR-7977 using HiPerFect Transfection Reagent (QIAGEN). Transfected AML cells were cocultured with MSCs in non-contact condition for 3 days. MSCs were transferred onto Lab-Tek II Chamber Slides (Thermo Scientific, Waltham, MA), and visualized using ZEISS/ELYRAS.1LSM780 confocal microscope (ZEISS, Oberkochen, Germany).

Luciferase assay with 3'-UTR of PCBP1 or JAG

Reporter plasmids that expressed luciferase with 3'-UTR of PCBP1 (SC216583) or JAG (SC205517) under the control of the SV40 promoter were purchased from OriGene Inc (Rockville, MD). The parental luciferase reporter plasmid (pMirTarget plasmid) was used as a control. Expression plasmid of human miR-7977 (MIR7977-MicroRNA) and empty vector control (pCMV-MIR) were purchased from OriGene Inc. MSCs were transfected with 2 μg of the luciferase reporters, control pMirTarget plasmid (pMIR-Cont), PCBP1 3'-UTR cloned into pMirTarget (pLuc-PCBP1) or JAG1 3'-UTR cloned into pMirTarget (pLuc-JAG1), together with 2 μg of MIR7977-MicroRNA (pMIR-7977) or pCMV-MIR (pMIR-Cont) using Lipofectamine LTX transfection reagent (Life Technologies). After transfection, the cells were incubated at 37°C and 5% CO_2 for 48 hrs, and then luciferase activities were measured according to the manufacturer's recommendation (Promega, Madison, WI) using a lumat LB 9507 luminometer (Berthold Technology, Bad Wildbad, Germany. Effects of miR-7977 on PCBP1 and JAG1 are presented as the ratio of the normalized value to the light emission observed in the cells transduced with the pMIR-Cont vector.